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REVIEW ARTICLE

ANTIBODY DRUG CONJUGATES: A LEAP AHEAD IN CANCER TREATMENT

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ABSTRACT

Monoclonal antibody (MAb) based targeted therapies have achieved appreciable success in various branches of drug therapeutics, predominantly when used along with cytotoxic drugs. These immunological therapies based on antibody-drug conjugates (ADCs) have been recently encouraged by the US Food and Drug Administration to treat Solid tumours, Melanoma, Breast Cancer and Hodgkin's lymphoma. Antibody drug conjugates (ADCs) are an important division of therapeutics that allows the antigen-selective ability of MAbs to deliver highly potent cytotoxic drugs at the site of antigen-expressing tumor cells. The use of MAb directed delivery can confer a high therapeutic index to highly potent cytotoxic drugs, increasing both the efficacy and safety of therapy. In other words, to achieve the goal of highly improved therapeutic efficacy and reduced toxicity, each component of an ADC i.e. the MAb, linker and the drug needs to be considered in context of targeted antigen. Furthermore, the mechanism of ADCs, characteristics of targets, methods of preparation, linker drugs being used in ADC design and regulatory requirements for new drug approval are discussed.
Keywords: Antibody-drug conjugates, Cytotoxic drugs, Monoclonal antibodies, Tumour

INTRODUCTION

Recent advances in oncology have focused on identifying drugs with improved selectivity for malignant cell versus normal cell as means to improve both the efficacy and tolerability of cancer treatment. One approach for enhancing and improving selectivity is to identify therapeutic targets with altered levels of expression on malignant versus normal cells and direct therapy against those targets. The introduction of monoclonal antibody (MAb) technology by Kohler and Milstein in 1975[1] led to thorough efforts to develop MAbs as highly selective antitumor therapeutics; however the immunogenicity of the very first generation, murine MAbs limited their application as therapeutics. The ability to obtain fully human MAbs from transgenic mice and by phage display has further enhanced and elaborated the clinical potential of these approaches [2–4]. There are currently 9 unconjugated MAbs approved by the FDA as cancer therapeutics. These MAbs include 2 chimeric, 4 humanized and 3 fully human monoclonal antibodies that display antitumor activity via blocking ligand/receptor interactions, or induce cell killing by means of antibody dependent cellular cytotoxicity (ADCC), or complement dependent cytotoxicity (CDC) [5]. Monoclonal antibodies have also been used to selectively deliver radionuclides [6,7], plant and bacterial toxins, [8–10] and a large variety of cytotoxic drugs [11–14]. MAb directed delivery of cytotoxic drugs is an area of intense and keen interest and there are currently at least 25 antibody drug conjugates (ADCs) undergoing clinical evaluation in oncology.

CHARACTERISTICS OF ANTIBODY DRUG CONJUGATES (ADC'S)

Antibody drug conjugates comprises of a MAb chemically coupled to a linker and a cytotoxic drug (Figure1). Mechanistically, ADCs are developed to be stable in circulation and to effect intracellular drug release followed by antigen-specific binding and internalization of the ADC. Currently the designing of ADCs as therapeutics has been focusing almost exclusively on the treatment of cancer. In contrast to small molecule cancer agents or function blocking MAbs, the targets for ADCs do not need to be causal in tumor progression. Rather those target antigens need to be most differentially expressed on the cell surface of malignant cells relative to normal tissues. The MAbs employed in first generation ADCs have identified cell surface antigens with different levels of tumor selectivity and also included MAbs that internalized following antigen binding and those that did not. To be highly effective, non-internalizing ADCs needed to remain intact into the circulation i.e. not release the hold of drug before reaching the target site and yet selectively release active drug at the tumor specific site. Typically, these ADCs made use of peptidyl linkers which were designed to be cleaved by enzymes like cathepsins and matrix metalloproteinases expressed dominantly at the tumor site, or linkers that would be releasing the drug by hydrolysis at a slightly acidic pH as observed in many solid tumors.

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For most of the part, these non-internalizing ADCs did not show much significant antigen-specific activity and did not significantly improve the therapeutic index (maximum tolerated dose/active dose) in contrast to that of the free drug [15,16]. The use of the MAbs that cause internalization following antigen binding has led to the designing of linkers that are stable in circulation and efficiently release the active drug following antigen specific binding, internalization and trafficking to endosomes/lysosomes [17–19]. Internalizing of ADCs has demonstrated a highly impressive preclinical [20–23] and clinical [24–29] activity.

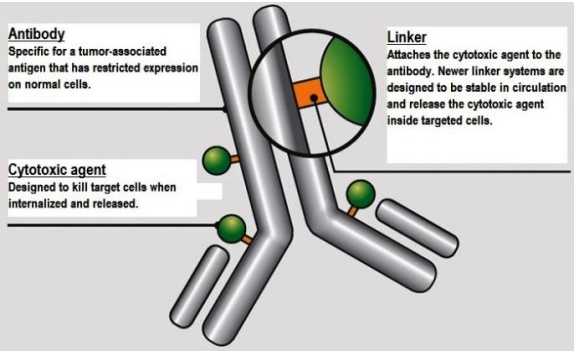

Figure 1: Schematic illustrating an Antibody Drug Conjugate (ADC)

ADCS: PHARMACOKINETIC ADVANTAGE VERSUS CHEMOTHERAPY

Traditional chemotherapy employs potent small molecules to destroy rapidly dividing cells, often through antimitotic or DNA-hampering mechanisms. Systemic administration of these drugs results in not only tumor killing and also damaging the healthy cells. The balance between these 2 actions plays a limiting factor in the efficacy and tolerability of single-agent chemotherapy. As a result, most of the cancer regimens consist of combinations of chemotherapeutic agents, each one of them administered at or near the maximum tolerated dose and for a very limited duration due to their uptake and leading to cumulative damage to normal tissues [30]. The rapid clearance of these small molecules and increase in hydrostatic pressure in the solid tumors has further decreased the tumor-specific activity of chemotherapy. In contrast, monoclonal antibodies, are large molecules (150 kDalton) that can be effectively retained in the vasculature for about several weeks and slowly diffuse into the perivascular tissue [31,32].The complementarity-determining regions can efficiently provide high-affinity binding which is directed against cell-surface antigens on tumor cells. The combination of a long half-life, specificity for tumor cells and high binding affinity results in the accumulation of antibody at the tumor site over a period of time. The lack of direct and serious cytotoxicity often facilitates prolonged treatment that is well tolerated and relatively safe. However, most monoclonal antibodies have very limited single-agent activity against cancer cells and are frequently used in combination with chemotherapy. Despite of a long time of active research and development, only 9 naked antibodies directed at 6 molecular targets have currently been approved by the US Food and Drug Administration (US-FDA) for cancer therapy (Table 1) [31]. ADCs are fabricated to take advantage of both the potent cell-killing activities of small molecules and the pharmacokinetic and bio-distribution potential of monoclonal antibodies.[19] ADCs have empowered antibodies by chemically conjugating a cytotoxic payload that can be effectively deliver and release that cytotoxic drug at the tumor while limiting systemic exposure to the cytotoxic agent. The proposed mechanisms of action for an ADC include; antibody engagement with a cell-surface target on cancerous cells, internalization and intracellular accumulation of the intact macromolecule to the lysosomes, rapidly releasing of the cytotoxic agent, and finally leading to efficient degeneration of tumor cells.

Table 1: Unconjugated monoclonal antibodies approved for cancer

Target	Antibody	Therapeutic Indication	First US Approval
CD20	Rituximab	NHL	1997
CD20	Ofatumumab	CLL	2009
Her2	Trastuzumab	Breast Cancer	1998
Her2	Pertuzumab	Breast Cancer	2012
CD52	Alemtuzumab	CLL	2001
EGFR	Cetuximab	Colon Cancer	2004
EGFR	Panitumumab	Colon Cancer	2006
VEGF	Bevacizumab	Colon Cancer	2004
CTLA-4	Ipilimumab	Melanoma	2011

There are specifically 5 important elements in the designing of effective ADCs: (1) The Molecular Target; (2) The Delivery Vehicle (monoclonal antibody or alternative scaffold); (3) Chemical Conjugation (method, site, and stoichiometry); (4) The Linker, including the suitable mechanism of drug release; and (5) The Cytotoxic Agent or Payload [34,35]. Current concepts for each of these elements are explicitly addressed in this review.

MECHANISM OF ACTION

A successful ADC consists of a MAb - a versatile platform for anticancer therapy which is capable of binding to the surface of tumour cell-specific antigens [35]. These antigens include over-expressed B-cell surface proteins in non-Hodgkin's lymphoma (NHL) such as CD19, CD20, CD21, CD22, CD40, CD72, CD79b and CD180, extending to the T-cell proteins CD25 and CD30

of the immune system. Moreover, proteins that are over expressed on carcinoma cells, including the human epidermal growth factor receptor 2 (HER2); prostate-specific membrane antigen (PSMA) and cryptic family protein 1 B (Cripto) are also antigens. These tumour-associated antigens have been studied as potential treatments for the following oncology indications: leukemia, lymphoma and multiple myeloma [36]. The function of cytotoxic drugs (e.g. auristatins, maytansinoids and calicheamicins), are designed to induce tumour cell death, by causing irreversible DNA damage and/or interfering with the mechanism of cell division [37]. The theory behind the mechanism of action of ADCs (Figure 2) involves the following processes: **Binding (Stage 1)** - The MAb component of the ADC binds to the target antigen on the surface of the tumour cell to produce an ADC-antigen (ADC-CDX) complex, which is engulfed into a clathrin-coated vesicle; **Clathrin-Mediated Endocytosis (Stage 2)** - This binding then initiates a cascade of events, involving the internalization of the ADC-antigen clathrin coated vesicle into the tumour cell. Consequently, the vesicle loses its coat and enables the ADC-antigen complex to fuse with an early sorting endosome, to initiate the release of the antigen from the ADC. At this stage, the antigen may be recycled back to the cell membrane. Furthermore, the early endosome converts to a late endosome containing the ADC; **Degradation (Stage 3)** – The internalized ADC is transported through the late endosome pathway to the intracellular compartment of a lysosome, where it is degraded to release the cytotoxic drug. The cleavable linkers rely on processes inside the cell to liberate the cytotoxic drug such as reduction of disulfide bonds mediated by glutathione (GSH) in the cytoplasm, exposure to acidic conditions (pH ~4) in the lysosome, or cleavage by specific proteases within the cell. Conversely, non-cleavable linkers require catabolic degradation [38] of the Mab, to release the cytotoxic drug retaining the linker and amino acid (lysine) residue, by which it was attached to the MAb; **Release (Stage 4)** – The cytotoxic drug enters the cytoplasm, where it binds to its molecular target. In **route A**- calicheamicin based drugs [39] interact with the minor groove of DNA and in **route B** -auristatins and maytansinoids disrupt the microtubules [40]. Subsequently, the cytotoxic drug may also pass through the cell membrane and enter other cells in close proximity thereby mediating a bystander killing effect; **Stage 5 – Cell Death:** The interaction of the cytotoxic drug with DNA and microtubules initiates a chain of events leading to apoptosis [41].

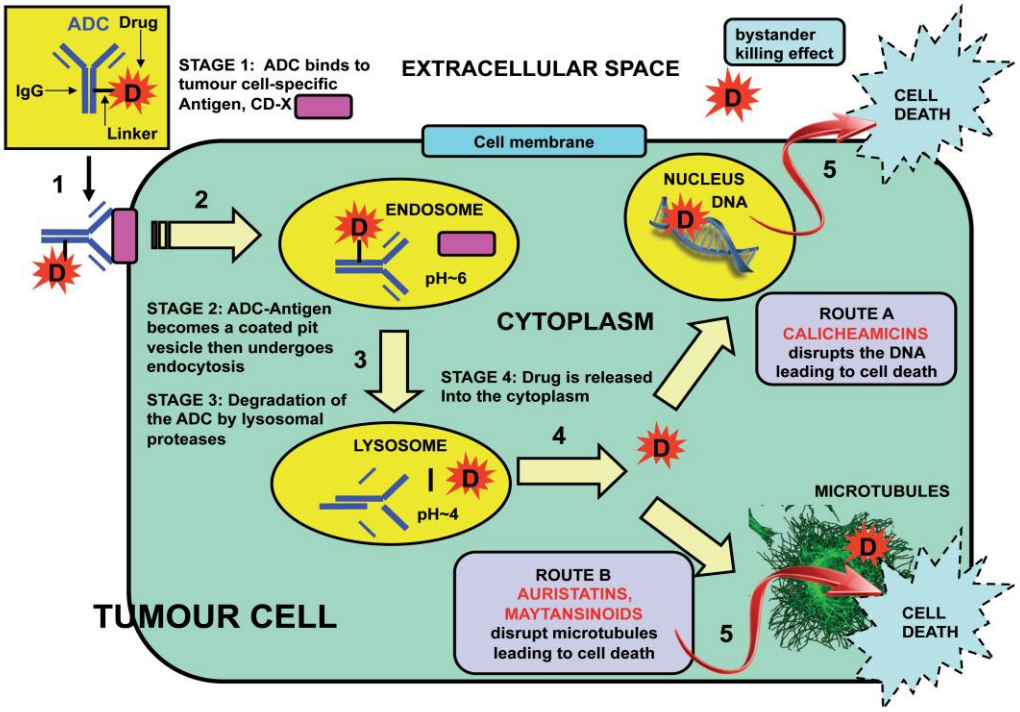

Figure 2: Schematic representation of the ADC-Antigen internalization process

VARIOUS AVAILABLE LINKERS AND DRUGS

To be sufficiently and desirably effective, an ADC must selectively bind, internalize and deliver an adequate intracellular concentration of drug that is sufficient to result in cell death and cancer cell degeneration (Figure 2). While in general, the conjugation strategies and methodologies used in ADC designing should have minimal effects on MAb affinity but still there are limited data available that can be used to define the optimal, or even the minimal, affinity that is required for an effective ADC fabrication. Rather than MAb affinity being the driver of ADC efficacy it is likely that the selectivity, efficiency of internalization and intracellular accumulation of a given MAb in composite will define an efficacious, potent and safe ADC. The copy number and heterogeneity of antigen expression must be considered in the selection of drug and linker. This is particularly very important for antigens expressed heterogeneously within a tumor where ADCs with local bystander activity [42,43] may be particularly be essentially desirable. The linker should be suitably stable in

circulation to facilitate the long circulating half-life of the MAb and yet release active drug following antigen-mediated internalization. Linkers can be broadly classified on the basis of their mechanism of drug release. Cleavable linkers release drug by hydrolysis or enzymatic cleavage following internalization whereas non-cleavable linkers, release drug via degradation of the MAb into lysosomes following antigen-specific internalization [17,44-47]. In addition to the mechanism of drug release, the specific site of conjugation, the potency of the drug and the average number of drug molecules per antibody needs to be carefully considered in the selection of the linker. Early ADCs incorporated drugs such as methotrexate [48-50], vinblastine [51,52] and doxorubicin [11,18,53,54] each of which had displayed clinical activity as free drugs. In general these ADCs have demonstrated antigen-specific activity *in vitro* and *in vivo* but they required high dose levels of ADC to achieve substantial and appreciable antitumor activity. A variety of approaches have been evaluated to increase the potency of these ADCs including increasing the quantity of drug delivered per MAb. In the case of doxorubicin conjugates, increasing the drug:MAb ratio over a range of 1–25 molecules of drug/MAb was achieved by direct conjugation [55], the use of branched linkers [56,57] or polymeric carriers [58].

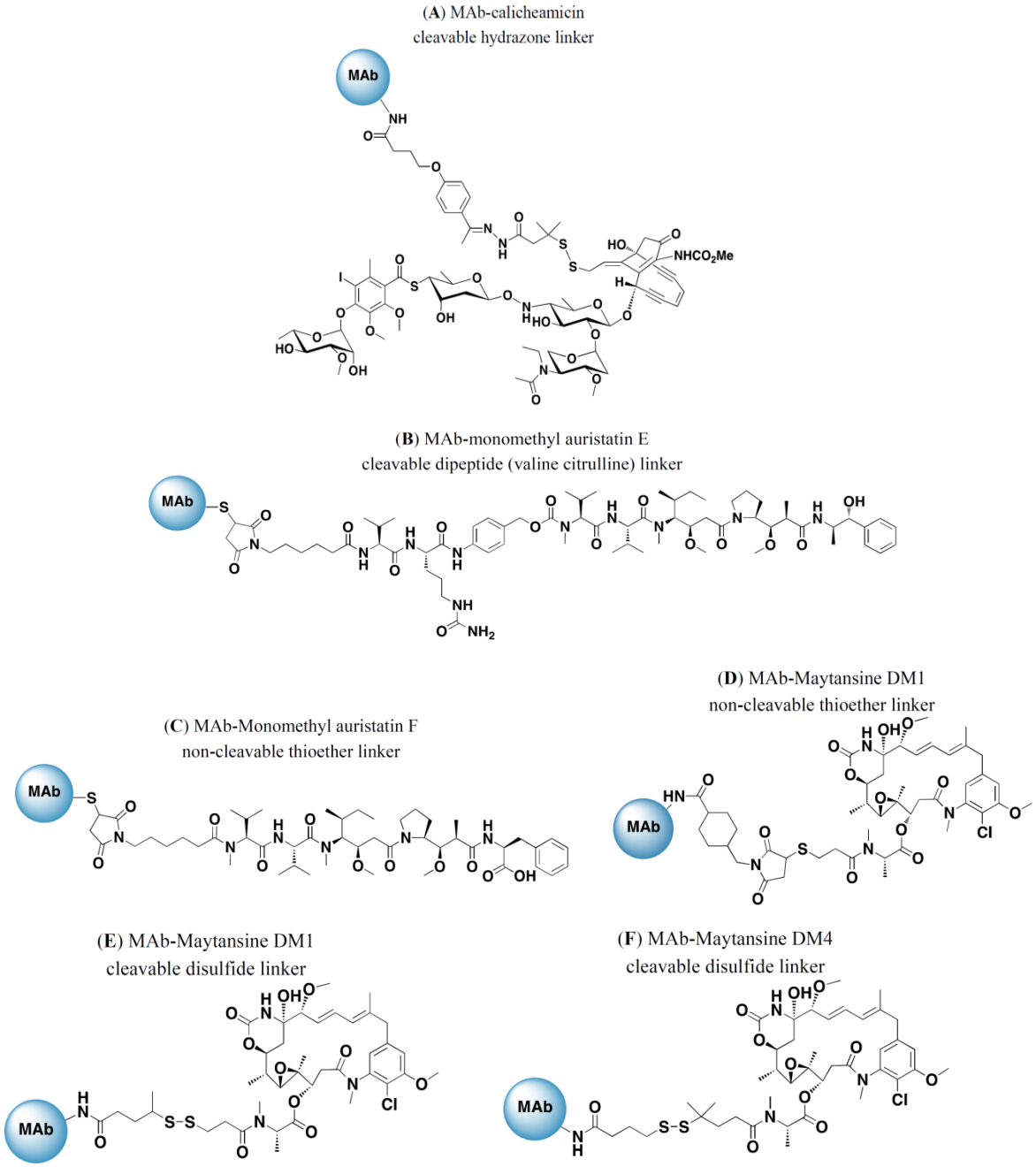

Figure 3: Various Linkers used in Antibody Drug Conjugates, (A)- MAb calicheamicin cleavable hydrazone linker; (B)- MAb-monomethyl auristatin E cleavable dipeptide (valine citrulline) linker; (C)- MAb-Monomethyl auristatins F non-cleavable thioether linker; (D)-MAb Maytansine DM1 non-cleavable thioether linker; (E)- MAb Maytansine DM1 cleavable disulfide linker; (F)-MAb Maytansine DM4 cleavable disulfide linker.

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Table 2: Antibody Drug Conjugates in Clinical Development

Candidate (Target Antigen)	Antibody-Drug Conjugate [Mab]-[Linker]-[Drug]	Oncology Indication	Developer
Phase lll of clinical development			
Inotuzumab ozogamicin (CD22)	[Hz IgG4]-[Hydrazone]-[Calicheamicin]	NHL	Pfizer
Gemtuzumab ozogamicin (CD33)	[Hz IgG4]-[Hydrazone]-[Calicheamicin]	Relapsed AML	Pfizer
Phase ll of clinical development			
Lorvotuzumab mertansine (CD56)	[Hz IgG1] - [SPP] - [Maytansine DM1]	Solid Tumours,MM	ImmunoGen
Glembatumumab vedotin (GPNMB)	[Hu IgG2] - [Valine-citrulline] - [Auristatin MMAE]	Breast Cancer, Melanoma	Celldex Therapeutics
SAR-3419 (CD19)	[Hz IgG1] - [SPDB] - [Maytansine DM4]	NHL	Sanofi
PSMA ADC (PSMA)	[Hu IgG1] -[Valine-Citruline]- [Auristatin MMAE]	Prostate Cancer	Progenics
RG7593/DCDT2980S (CD22)	[Hz IgG1] - [Valine-Citruline]- [Auristatin MMAE]	NHL	Genentech Roche
RG-7596 (CD79b)	[Hz IgG1] -[Valine-Cituline] - [Auristatin MMAE]	NHL	Genentech Roche
BT-062 (CD138)	[Ch IgG4] - [SPDB] - [Maytansine DM4]	MM	Biotest
Phase l of clinical development			
SGN-75 (CD70)	[Hz IgG1] -[Malemidocaproyl] -[Auristatin MMAF]	NHL,RCC	Seattle Genetics
BAY 79-4620 (CA-IX)	[Hu IgG1]-[Valine-citruline]-[auristatin MMAE]	Solid Tumours	Bayer
Milatuzumab doxorubicin (CD74)	[Hz IgG1] -[Hydrazone]- [Doxorubicin]	MM	Immunomedics
AGS-5ME (SLC44A4)	[Hu IgG2] -[Valine-Citruline]-[Ausistatin MMAE]	Pancreatic, Prostate Cancer	Astellas
BAY 94-9343 (Mesothelin)	[Hu IgG1] - [SPDB]-[Maytansine DM4]	Solid Tumuors	Bayer
ASG-22ME (Nectin-4)	[Hu IgG1] -[Valine-Citruline] -[Auristatin MMAE]	Solid Tumours	Astellas

Abbreviations-Ch:chimeric;Hz:humanized;hu; fully human;MMAE:monomethyl auristatins E;MMAF:monomethyl auristatinF;NHL;non-Hodgkin's Lymphoma;PSMA:Prostate - Specific Membrane Antigen; RCC:Renal Cell Carcinoma; GPMNB:Glycoprotein NMB;AML:Acute Myeloid Leukaemia; MM:Multiple Myeloma;CRC:Colorecta Carcinoma. (Source: www.clinicaltrials.gov, 2013).

PREPARATION OF ANTIBODY DRUG CONJUGATES (ADC'S)

Figure 4 displays a generic process overview of various process steps involved in ADC manufacturing using a non-cleavable Succinimidyl-4-(N-maleimidomethyl) cyclohexan-1-1carboxylate (SMCC) linker. The Succinimidyl-4- (N-maleimidomethyl) cyclohexan-1-carboxylate (SMCC) linker is an amine-to-sulfhdryl crosslinker that comprises of NHS-ester and maleimide reactive groups located at opposite ends of a cyclohexan-stabilized spacer arm. The NHS esters react with the primary amines at pH 7-9 to form suitable stable amide bonds. Maleimide reacts with sulhydryl groups at a pH of 6.5-7.5 to form stable thioether bonds. The maleimide group of SMCC is stable up to pH 7.5 because of the presence of cyclohexane bridge in the spacer arm [59,60].

ADC production process utilizing the SMCC linker is characterized by steps which involve controlling the antibody modification (preparing the antibody for the conjugation reaction) and conjugation reaction (introduction of drug moiety) employed to achieve the desired level of drug loading. The molar ratio of drug to antibody can be adjusted by changing the reaction stoichiometry to deliver the desired level of potency to the target tissue [61]. Additional steps such as removal of process related contamination, concentration of the

active pharmaceutical ingredient (API) and stabilization of the resulting bulk drug substance (BDS) are also critical steps in the manufacturing process.

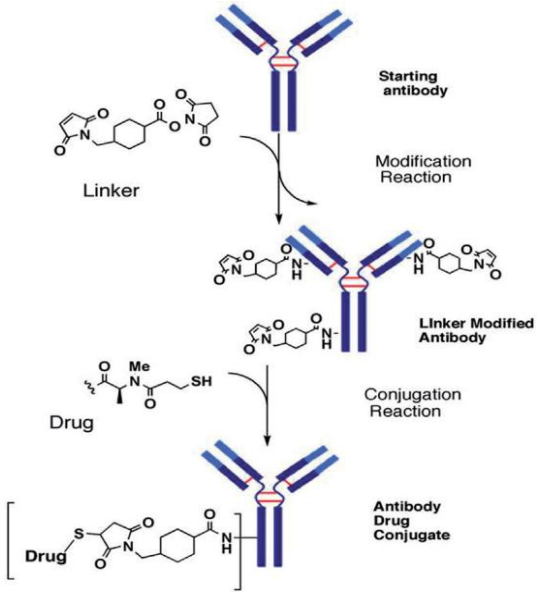

Figure 4: ADC Prepartion Process using non-cleavable Succinimidyl-4-(N-maleimidomethyl) cyclohexan-1-1carboxylate (SMCC) linker.

REGULATORY ASPECTS

In order to develop and characterize the ADC certain analytical methods must be implemented in order to verify and identify the type of MAb and cytotoxic drug to be used in its manufacture [62]. These analytical techniques are used for the characterization of the ADC and may include protein mass spectrometry (PMS) and capillary electrophoresis (CE). A wide range of analytical tools can be effectively employed to determine the molecular weight of the ADC including peptide mapping and sequencing. The structure and linkage of the linker-drug combination can further be determined and analyzed using multi-NMR [63] and FTIR spectroscopic techniques [64]. X-Ray crystallography can further be used to assess and examine the peptide or antibody structure and the drug to antibody ratio (DAR) can be suitably evaluated using UV methods [65]. Subsequently, the application of size-exclusion chromatography (SEC) techniques can be used to determine fragmentation pattern and aggregate patterns during the synthesis of the ADC [66]. Furthermore, the antigen binding and biological activity of the MAbs must also be assessed against ELISA, *in vitro* cell-based assays and *in vivo* studies [67]. A critical factor which needs attention is to develop robust analytical methods to determine the level of free cytotoxic drug [68]. In addition, chemical impurities obtained during the synthesis which include the impurity profile from host cell proteins must also be identified and characterized [69]. The manufactured ADC must be evaluated as a new molecular entity and not as a separate product (antibody-linker-drug). This is to elucidate a structure/function relationship towards: the pharmacokinetics profile and low immunogenicity; the cytotoxic drug must demonstrate potent anti-tumour activity; linker has to be stable so as to enable the delivery of the ADC to target antigen; MAb must have high affinity and selectivity towards the cellular targets. The tumour-associated antigen expression ratio must be significantly high in tumours in comparison to normal tissue and must allow the ADC-antigen complex to be internalized [70].

Table 3: Merits & De-merits of ADC therapy

Merits of ADC Therapy	De-merits of ADC Therapy
Selective delivery of cytotoxic drugs to tumour cells	Molecular targets having similar expression may also get exposed to the dug leading to toxicity
Specific binding to target antigen	Requires screening of antigen of interest
Large therapeutic index	Premature release of cytotoxic drug may lead to lethal effects
Stability of conjugate ensures extended and prolonged circulation half life	Sufficient concentration may not be achieved at target site
Reduction of adverse effects	Heterogeneous antigen expression can hamper the desired results

(Source: Beverly A.Teicher; Ravi V.J. Chari; Clin Cancer Res; 2011; 17(20); 6389–97.)

PRESENT AND FUTURE OF ADC'S

Currently, there are 2 ADCs available for patients in the United States. However, with more than about 30 additional molecules under clinical trials (Table 2), it is very likely that number of approved ADCs will enhance substantially in the coming decade. Moreover, this class of drugs provides a new opportunity to re-examine the future and potentially safe cytotoxic therapy. The combination of improved and enhanced potency with better tolerability profile offers the ray of hope for curing more life threatening cancers and, for those cancers that cannot be totally eradicated, ensuring an extended therapy and an improved quality of life for these patients. A century after Paul Ehrlich, his challenge has been taken up by a new generation of scientists who are working deligently to improve the specificity and activity of cancer chemotherapy. Although ADCs have just recently come up in the scenario, the evolution of the field is rapidly accelerating and the impact on cancer care is likely to be great in the years to come.

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